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Evaluation of the antiproliferative activity of three types of *Annona muricata* extract in the traditional treatment of cervical cancer induced in Wistar rats

Agossou Esse, Akotegnon Rodrigue, Savoeda Perside, Behanzin G. Justin and Sezan Alphonse *

Laboratory of Pharmacology and Improved Traditional Medicines, Department of Animal Physiology, Faculty of Science and Technology, University of Abomey-Calavi, BP 526 Cotonou, Republic of Benin.

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Abstract

This study was concerned with the evaluation of the antiproliferative activity of *Annona muricata* in the traditional treatment of cervical cancer induced in Wistar rats. The aim of the present work was to compare the effect of ethyl, dichloromethane and ethyl acetate extracts of *Annona muricata* leaves to determine the most effective one on cervical cell differentiation in wistar rats.

For this purpose, we made three extracts namely: ethyl extract, dichloromethane and ethyl acetate) from dried leaf powder of *Annona muricata*. The antiproliferative (anticancer) activity was evaluated by assaying the alkaline phosphatase and total protein parameters on cervical crushings of Wistar rats, in which cervical cancer was previously induced by exposure to Cycas revoluta extract through gavage of 100mg/kg of the ethyl extract of Cycas revoluta.

The antiproliferative (anticancer) activity was effective by administration of a dose of 100 mg/kg of ethyl, dichloromethane and ethyl acetate extracts of *Annona muricata* leaves. This activity showed a variation of intensity according to the type of extracts. Thus the dichloromethane extract was the most active.

Key words: *Annona muricata; Cycas Revoluta;* Cervical Cancer*;* Antiproliferative*;* Extracts*;* Differentiation.

1. Introduction

1.1. Problematics

Cervical cancer is a dreaded disease whose rate is increasing day by day. About 6 million new cases of cancer are reported annually worldwide (ABIODUN, et al 2011).

The therapeutic arsenal currently available to the CNHU (National University Hospital Center) of Benin against cancer is mainly composed of curative surgery and chemotherapy. However, signs of intolerance to chemotherapy treatment are observed in the digestive system (nausea and vomiting: 71.9% of cases), the hematological system (anemia: 89.1% of cases; leukopenia: 5.8% of cases, thrombocytopenia: 5.1% of cases) and the cutaneous system (alopecia: 3.3% of cases; pigmented nails: 2.9% of cases, pruritus: 22% of cases) (Edah. 1999). Moreover, whatever the therapeutic means used, its cost seems high in relation to the financial possibilities of the patients. Thus, the populations of Benin who generally live on a low income turn to palliative solutions, such as herbal medicine (Cazorla, 2014).

* Corresponding author: Sezan Alphonse

Our work consists in the study of *Annona muricata*, which is a plant very much used in the anticancer pharmacopoeia in Benin (Akoegninou, 2006). Its quasi-systematic use by phytotherapists in the treatment of cervical cancer has led us to verify the antiproliferative activity of three extracts of *Annona muricata* leaves and to determine the most effective extract. Thus, we will be able to contribute to the development and local production of traditional drugs with determined toxicity and efficacy at low cost.

2. Materials and Methods

2.1. Animal material

It consists of female Wistar rats that have reached puberty. They are divided into four (4) batches of four (6) rats.

2.2. Methods

2.2.1. Extraction Drying of the leaves

The fresh leaves of *Annona muricata* and Cycas revoluta were collected and identified by botanical experts of the National Herbarium of the University of Abomey-Calavi, then dried in the shade on a bench of the Laboratory of Biomembranes, and Cellular Signaling at the temperature of 16o C. After drying, the leaves were powdered with the help of a mill and preserved in glass jars in order to avoid the installation of polluting micro-organisms.

Figure 1 Powder of dried leaves of *Annona muricata* (Photo Perside SAVOEDA. 2021)

Figure 2 Powder of dried leaves of *Cycas revoluta* *(*Photo Perside SAVOEDA. 2021}

2.3. Ethyl extraction of Cycas revoluta

A mass of 100g of Cycas revoluta leaf powder is macerated in 1l of ethanol at 95° C, for 72 hours under agitation. Then the macerated is filtered through hydrophilic cotton fiber. The filtrate obtained is evaporated using the ROTAVAPOR evaporator at 40°C. The recovered extract is stored in a labelled glass bottle. The yield is determined by the ratio of the weight of the dry extract after evaporation to the weight of the dry plant material used for extraction multiplied by 100 (Medane 2012).

2.4. Preparation of ethyl, dichloromethane and ethyl acetate extracts of *Annona muricata*

A mass of 100g of leaf powder; is macerated in 1l of ethanol at 95° C, for 72 hours under agitation. Then the macerated is filtered through the hydrophilic cotton fiber. The filtrate obtained is evaporated using the ROTAVAPOR evaporator at 40°C. The recovered extract is stored in a labelled glass bottle. The yield is determined by the ratio of the weight of the dry extract after evaporation to the weight of the dry plant material used for extraction multiplied by 100 (Medane 2012).

On the previous residue, we add successively dichloromethane until total exhaustion, and ethyl acetate. The two recovered fractions are dried then weighed to determine the extraction yields.

a b c d

Figure 3 Extraction process on *Annona muricata* leaves

(a: powder mixed with ethanol 95°C under agitation; b: filtration; c: filtrate of the mixture of leaf powder/ethanol 95°C under evaporation with ROTAVAPOR; d: extract of *Annona muricata* leaves dried) (Perside SAVOEDA photograph. 2021)

2.5. Research of anticancer effects of *Annona muricata* leaf extracts

The animals were acclimatized to the laboratory conditions from birth. They were fed with protein-rich, lipid-rich pellets and had access to tap water in small jars without interruption and had free access to food and water. The breeding is carried out in a lit room and at room temperature.

We have four (04) batches of four (06) female rats each weighing 200g of body weight on average. Lot 1 is used as a control and the other lots are treated respectively by a dose of ethyl extract, dichloromethane and ethyl acetate of *Annona muricata*.

2.5.1. Extract Administration

The administration of the extract is carried out to rats by gavage with a gastric tube. The gavages are made in daily intake.

Gavage of rats with ethyl extract of Cycas revoluta

Oral gavage was performed for 14 days with an ethanolic extract of Cycas revoluta leaves (batch 2,3,4) see Table I.

Table 1 Distribution of rats per batch and per dose of *Cycas revoluta*

Batch 1 (control)	**Batch 2**	**Batch 3**	**Batch 4**
Granulate + distilled water	Pellet + water + 100mg/kg of ethanolic extract of Cycas revoluta dissolved in 1 ml of distilled water	Pellet + water + 100mg/kg of ethanolic extract of Cycas revoluta dissolved in 1 ml of distilled water	Pellet + water + 100mg/kg of ethanolic extract of Cycas revoluta dissolved in 1 ml of distilled water

2.6. Determination of the biochemical value

On the 14th day, two rats from each batch were sacrificed, dissected and the cervix was extracted (Fig. 2). The cervix previously rinsed in physiological solution; is ground in a mortar with 5ml of physiological solution and acid wash sand for 10mn at room temperature. The crushed material was centrifuged at 6000 rpm for 5 minutes. Total protein and PAL were tested in the supernatant obtained after centrifugation of the grind.

2.7. Gavage of rats with ethyl, dichloromethane and ethyl acetate extracts of Anonna muricata leaves

After the verification of the cell proliferation and differentiation stimulating activity of the ethanolic extract of Cycas revoluta leaves, the remaining rats in each batch were used for further experimentation. The control lot received only

distilled water and pellets throughout the experiment. Batches 2, 3 and 4 (Table II) whose rats had been fed during the first two weeks of the experiment with a diet composed of pellets and 100mg/kg body weight of Cycas revoluta extracts by gavage, received respectively 100mg/kg body weight of ethyl, dichloromethane and ethyl acetate extract of *Annona muricata* leaves by gavage

Table 2 Distribution of rats per batch and per dose of Anonna muricata extract

Batch 1 (control)	**Batch 2**	**Batch3**	**Lot4**
Granulated + distilled water (without Anonna muricata)	Pellet + 100 mg/kg body weight of ethanolic extract of A. muricata	Pellet + 100 mg/kg body weight of dichloromethane extract of A. muricata	Pellet + 100 mg/kg body weight of ethyl acetate extract of A. muricata

2.8. Verification of the antiproliferative effect of Anonna muricata extracts: determination of biochemical parameters

On the 28th day, the rats of the 4 batches were sacrificed, dissected and the cervix was extracted (Fig. 2). The cervix, previously rinsed in physiological solution, was ground in a mortar with 5 ml of physiological solution and acidic washing sand for 10 min at room temperature. The crushed material was centrifuged at 6000 rpm for 5 minutes. Total protein and PAL were tested in the supernatant obtained after centrifugation of the grind.

2.9. Techniques for the determination of different parameters

2.9.1. Total Protein

Principle

The total proteins form with the cupric ions, in alkaline medium a colored complex.

✓ Procedure

Table 3 Method of determination of total proteins

Measure in test tubes	**Assay**	**Standard**	**blank**
Sample	20µl		
Standard		20µl	
Sodium chloride	1ml	1ml	1ml
Biuret	1ml	1ml	1ml
Mix. Let for 10 mn between 20°C and 25°C. Read absorbances at 550nm (530-570) against the positive blank			

The calculation is based on the following rule:

(Abs.Dosage/ Abs. Standard) * concentration of the standard

Alkaline phosphatase

- Principle

The optimized method is based on the recommendations of the DGKC (Food Society of Clinical Chemistry, 1972) and the SCE (Scandinavian Society of Clinical Chemistry). In alkaline medium, alkaline phosphatases catalyze the hydrolysis of p-nitrophenylphosphate to p-nitrophenol and phosphate. The rate of appearance of p-nitrophenol, monitored by the change in absorbance at 405 nm, is proportional to the PAL activity in the specimen.

Table 4 Method of determination of alkaline phosphatase

Introduce in ther mostated tank with 1cm optical path	
Reagent	1ml
Let the temperature equilibrate at 37°C then add	
Specimen	
Mix. After 1mn read absorbance at 405nm , then each minutes for 3mn	
Calculate the absorbance variations average for each minutes (ΔAbs/min)	

The result is determined by the following formula: IU/L= (ΔAbs/min) × 5450

2.10. Statistical analysis

Graphs were plotted using excel software. The different averages were compared using the student t test. The level of significance was set at 5%.

3. Results

3.1. Extraction yields

The extraction yield of *Annona muricata* leaf and Cycas revoluta leaf extracts are recorded in Table V

Table 5 Extraction yields

Retrieved from	**Ethanolic extract of *A. muricata***	**Dichloromethane extract *A. muricata***	**Ethyl acetate extract of *A. muricata***	**Ethanolic extract Cycas revoluta**
Yield % (%) $R = \left(\frac{\text{Extract mass}}{\text{Leaves powder mass}}\right) \times 100$	8.36	2.87	2.69	15.86

Verification of the carcinogenic effect of Cycas powder

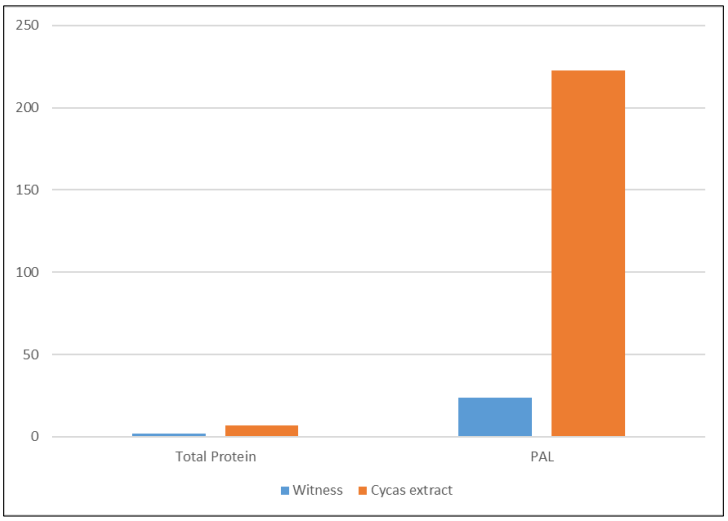

Figure 4 Variation of PAL and Total Protein parameters

The administration of Cycas revoluta extract for 2 weeks, i.e. 14 days, significantly influenced the variation of PAL and total protein parameters. Indeed, compared to the control rats that received only distilled water and a simple diet of pellets and tap water, an increase in PAL (P<0.013) and a slight increase in total protein (P<0.0

The ratio of PAL to total protein (Figure 5) of the Cycas extracted rats is higher than that of the control rats

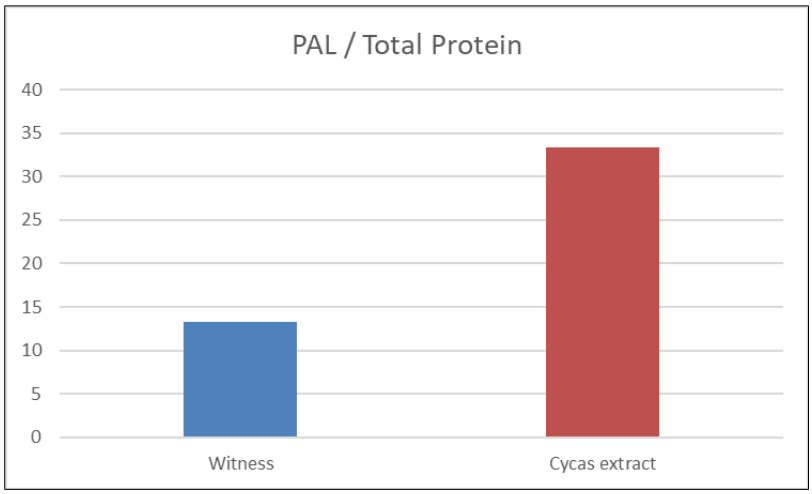

Figure 5 Change in the ratio of PAL to total protein

3.2. Verification of the pharmacological effect of extracts of *Annona muricata* leaves

After administration of the ethyl extract of *Annona muricata* leaves, there was a considerable decrease in PAL activity and total protein (Figure 6). After administration of the dichloromethane extract of *Annona muricata* leaves, there was a considerable decrease in PAL activity. After administration of the ethyl acetate extract of *Annona muricata* leaves, there was a considerable decrease in PAL activity.

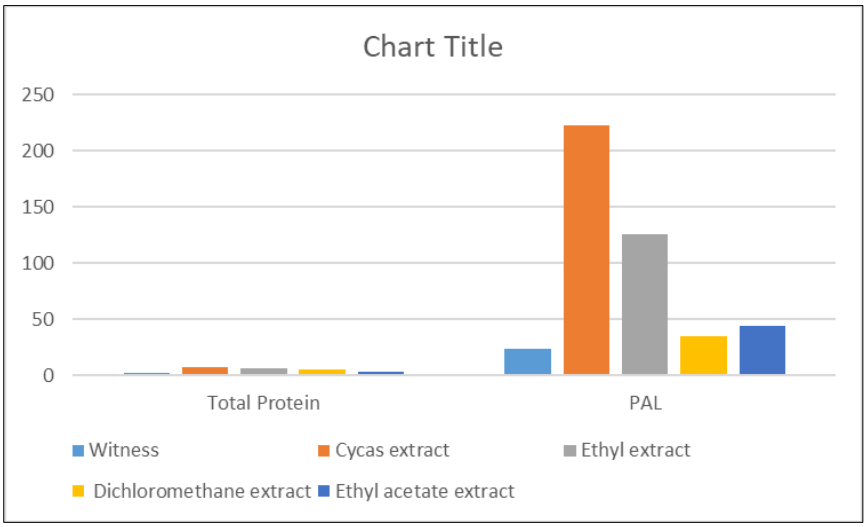

Figure 6 Variation of PAL and Total Protein parameters after administration of the extracts

Figure 7 presents the PAL/total protein ratio of each batch of rats that received the different extracts of *Annona muricata* compared to the control batch and Cycas revoluta ratio. It appears that the PAL/total protein ratio of rats that received the extracts decreased

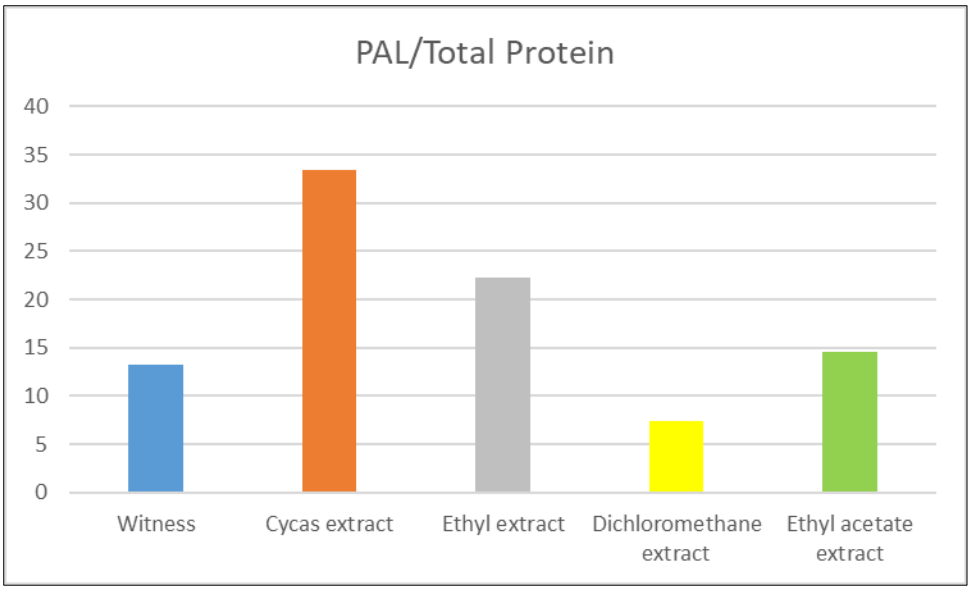

Figure 7 Variation in the ratio of PAL to total protein after administration of *Annona muricata* extracts

Figure 8 shows the PAL/total protein ratio of the different extracts of *Annona muricata* compared to the control batch. A drop in the PAL/total protein ratio was observed in rats treated with *Annona muricata*.

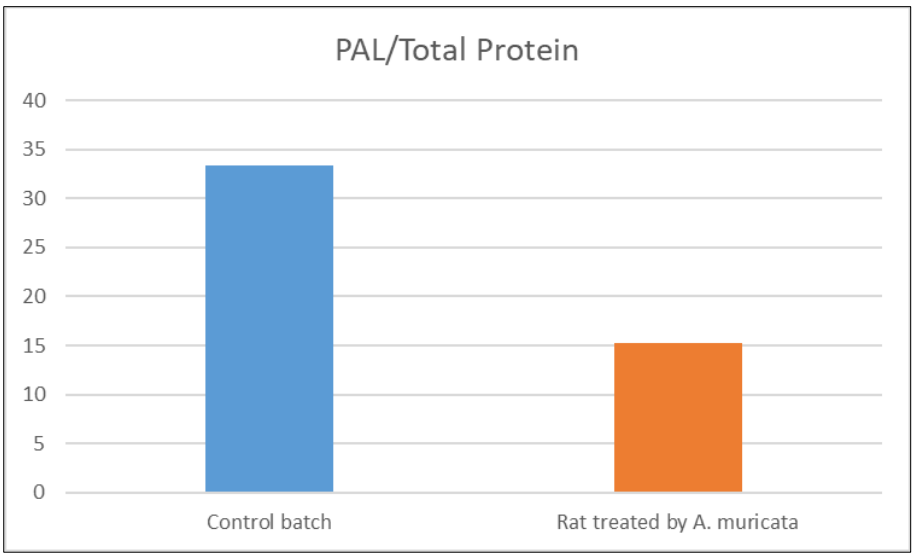

Figure 8 Comparison of the PAL/total protein ratio between the control batch (Cycas revoluta extract only) and the batch treated with *Annona muricata*

4. Discussion

Cancer is characterized by the progressive invasion of the organ of origin and then of the entire organism by cells that have become insensitive to the mechanisms of tissue homeostasis and have acquired an indefinite capacity to proliferate (Rappillard a. 2010). Cancer is a major cause of morbidity and mortality worldwide. According to the WHO in 2007, cancer is a term used to designate the autonomous and anarchic malignant proliferation of cells. In Africa, 600,000 cases of cancer occur each year and 500,000 patients die, according to WHO figures. (Ly, 2007). More specifically, cervical cancer is one of the new public health challenges for developing countries in sub-Saharan Africa. Cervical cancer ranks as the second most common gynecologic cancer in Benin (Tonato et al., 2013). Human papillomavirus (HPV) prevalence (21.5%) is also observed to be increasing in West Africa, and in increasingly younger patients (Monsonego, J. 2006).

In an extensive study, the anticancer properties of 187 plants were evaluated (Kintzios, 2012) among which *Annona muricata*. Badrie and colleagues (2009) and Ana and collobaroteurs (2016) claim that *Annona muricata* is a potent anti-cancer in the Annonaceae family. Studies conducted in Nigeria by Okolie Ngozi P. et al. showed an anticancer effect of ethyl extracts of *Annona muricata* leaves in colorectal cancer induced by Cycas circinalis which is a plant of the same family as Cycas revoluta . Indeed, this study conducted in Nigeria proved that the administration of a dose of 100mg/kg of rat weight of ethyl extracts of the leaves of *Annona muricata*, during 3 weeks, preceded by an induction of cancer by the consumption of food composed of 5% of the powder of Cycas circinalis; would induce a decrease of the cellular proliferation. He would attribute this anti-proliferative activity to acetogenins which are compounds contained in *Annona muricata*. Acetogenins would destroy cancerous cells by blocking the Nadh-CoQ oxidoreductase complex (complex 1), which supplies them with ATP. Acetogenins are also believed to have effective anti-oxidant properties. (Wamidh Hadi Talib. 2011)

Thus, our study proposed to verify the anti-cancer properties attributed to *Annona muricata* in the literature. We tested the anti-proliferative and anti-differentiation properties of ethyl extracts of *Annona muricata* leaves on cells from the cervix of Wistar rats, previously exposed to Cycas revoluta, which is a known carcinogenic plant.

Thus we performed an ethyl extraction using the powder of *Annona muricata* leaves and ethanol 95°C. We have at the end of this extraction obtained a yield of 8.36%, which we consider average compared to the yield obtained by Eka Prasati et al. (2012) which is 14.86%. This difference would be explained by the difference in the degree of ethanol used which is 70° in their case.

Cycas revoluta contains cycasin (aglycone methyl azoxymethanol), which is known to induce liver cancer and many others (Anil Kumar. 2012). After ingesting a meal containing Cycas powder, intestinal bacteria hydrolyze the glucoside linkage of cycasin to release the methyl azoxymethanol aglycon (Laqueur G.L. (1965 and Wamidh Hadi Talib. 2011). Since researchers realized the effective carcinogenic properties of methyl azoxymethanol, these agents have been used to create reliable cancer animal models (to induce cancers) (Weischselbaum, TE.1946). It is with this in mind that we also used the powdered leaves of Cycas revoluta, which is a plant of the same family as Cycas circinalis found in Benin, to induce cancer in our study.

We have therefore induced this carcinogenicity of Cycas revoluta leaf powder by means of a test of exposure of rats to Cycas revoluta powder and the dosage of PAL and total proteins. It should be noted that the PAL parameter was chosen because it is a cellular enzyme found at different levels of the cell, and its level reflects the state of the tissue, since an increase in this level would be a sign of tissue suffering. As for total proteins, which are the result of the transcription of certain genes, their sudden increase could reflect abundant and abnormal transcription and could characterize the carcinogenesis of the organ concerned (the cervix).

We obtained as a result of this experimentation, that compared to control rats that received during the experimentation only distilled water accompanied by a simple diet consisting of pellets and tap water an increase in PAL and total protein levels. This is consistent with the results of Okolie Ngozi P. et al 2013, HOUNDEFFO Tiburce et al 2017.

This increase in the rate of PAL and total protein parameters in rats having consumed Cycas revoluta powder would be explained by a greater production of Succinate from the Krebs cycle which is the logical continuation of glycolysis and which is also known as an intermediary between the mitochondrial dysfunction perceived in cancer cells and oncogenesis via the HIF-α factor. It would augur an abnormal proliferation of the cells of the cervix of these rats, which allows us to verify the hypothesis of the carcinogenicity of the powder of the leaves of Cycas revoluta.

Kouadio et al, (2006), points out that the PAL/total protein ratio accounts for the stage of differentiation. Our results show a higher PAL/total protein ratio in the sample rats compared to the controls. These results suggest that Cycas revoluta also stimulates differentiation in cervical cells.

The second phase of our tests concerned the study of the anti-proliferative and anti-differentiation activity of ethyl extracts of *Annona muricata* leaves on cervical cancer cells of Wistar rats previously exposed to Cycas revoluta extract. Our results show that after administration of *Annona muricata* leaf extracts, there is a decrease in total protein and PAL levels, compared to rats only exposed to Cycas revoluta. In addition, the PAL/total protein ratio of rats given Cycas extracts followed by *Annona muricata* extracts decreased significantly. These results are consistent with those of Okolie et al. in 2013, who had shown anti-cancer activity of ethyl extracts of *Annona muricata* leaves through anti-proliferative pharmacological properties on cancer cells. From our results, it results that the ethyl extracts of *Annona muricata* leaves could possess anti differentiation and anti proliferative properties on Wistar rat cervical cells. Then it was observed that compared to batch 2 and 4, the rats of batch 3 having received as treatment during the 4 weeks of experimentation

a diet containing the cycas extract and a daily gavage with our dichloromethane extracts at the dose of 100mg /kg of rat weight underwent a much more significant decrease of the PAL/total protein ratio. Thus the dichloromethane extracts would be more effective than the other extracts. Finally, the difference in the effects of *Annona muricata* leaf extracts according to the nature of the extract could be explained by the fact that the extracts contain different active biomolecules.

5. Conclusion

The results of our study show that Cycas revoluta extract significantly stimulates the differentiation of Wistar rat cervical cells and dichloromethane extracts of *Annona muricata* leaves much more significantly decrease the differentiation. These results suggest research into the mechanisms of action of these extracts and the biomolecules they contain.

Compliance with ethical standards

Disclosure of conflict of interest

All authors in the making of this scientific article have no conflict of interest.

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